Exercise training in patients with chronic heart failure promotes restoration of HDL functional properties

Adams, Volker ; Besler, Christian ; Fischer, Tina ; Riwanto, Meliana ; Noack, Friederike ; Höllriegel, Robert ; Oberbach, Andreas ; Jehmlich, Nico ; Volker, Uwe ; Winzer, Ephraim B ; Lenk, Karsten ; Hambrecht, Rainer ; Schuler, Gerhard ; Linke, Axel ; Landmesser, Ulf ; Erbs, Sandra

Abstract: Rationale: HDL exerts endothelial-protective effects via stimulation of endothelial cell (EC) NO production. This function is impaired in patients with cardiovascular disease. Protective effects of exercise training (ET) on endothelial function have been demonstrated Objective: This study was performed to evaluate the impact of ET on HDL-mediated protective effects and the respective molecular pathways in patients with chronic heart failure (CHF). Methods and Results: HDL was isolated from 16 healthy controls (HDLhealthy) and 16 patients with CHF-NYHA-III (HDLNYHA-IIIb) before and after ET, and 8 patients with CHF-NYHA-II (HDLNYHA-II). EC were incubated with HDL and phosphorylation of eNOS-Ser(1177), eNOS-Thr(495), PKC-ßII-Ser(660) and p70S6K-Ser(411) was evaluated. HDL-bound malondialdehyde and HDL-induced NO production by EC were quantified. Endothelial function was assessed by flow-mediated dilatation (FMD). The proteome of HDL particles was profiled by shotgun LC-MS/MS. Incubation of EC with HDLNYHA-IIIb triggered a lower stimulation of phosphorylation at eNOS-Ser(1177) and a higher phosphorylation at eNOS-Thr(495) when compared to HDLhealthy. This was associated with lower NO production of EC. In addition an elevated activation of p70S6K, PKC-ßII by HDLNYHA-IIIb, and a higher amount of malondialdehyde bound to HDLNYHA-IIIb compared to HDLhealthy was measured. In healthy individuals ET had no effect on HDL function, whereas ET of CHF-NYHA-IIIB significantly improved HDL function. A correlation between changes in HDL-induced NO production and FMD improvement by ET was evident. Conclusions: These results demonstrate that HDL-function is impaired in CHF and that ET improved the HDL-mediated vascular effects. This may be one mechanism how ET exerts beneficial effects in CHF.

DOI: https://doi.org/10.1161/CIRCRESAHA.113.301684

Exercise Training in Patients With Chronic Heart Failure Promotes Restoration of HDL Functional Properties

Volker Adams, PhD[1,*], Christian Besler, MD[2,*], Tina Fischer, BS[1], Meliana Riwanto, BS[2], Friederike Noack, MS[1], Robert Höllriegel, MD[1], Andreas Oberbach, MD[3], Nico Jehmlich, PhD[4], Uwe Völker, PhD[4], Ephraim B. Beck, MD[1], Karsten Lenk, MD[1], Rainer Hambrecht, MD[5], Gerhard Schuler, MD[1], Axel Linke, MD[1], Ulf Landmesser, MD[2], Sandra Erbs, MD[1]

(1) University Leipzig – Heart Center Leipzig, Department of Cardiology, Leipzig, Germany
(2) University Hospital Zürich, Department of Cardiology, Zürich, Switzerland
(3) University Leipzig, Heart Center Leipzig, Department of Cardiac Surgery, Leipzig, Germany
(4) Ernst-Moritz-Arndt-University Greifswald, Interfaculty Institute for Genetics and Functional Genomics, Department of Functional Genomics, Greifswald, Germany
(5) Heart Center Bremen, Klinikum Links der Weser, Bremen, Germany

* both authors contributed equally to the manuscript

Running title: Exercise training in CHF and HDL function

Total word count: 6486

Address for correspondence:
Volker, Adams, PhD
Department of Cardiology
Heart Center Leipzig, Leipzig University
Strümpellstrasse 39, D-04289, Germany
04289 Leipzig
Germany.
(Tel) +49-341 865 1671
(Fax) +49-865 1461
e-mail: adav@medizin.uni-leipzig.de

Abstract:

Background: HDL exerts endothelial-protective effects via stimulation of endothelial cell NO production. This function is impaired in patients with cardiovascular disease. Studies demonstrated that exercise training (ET) has protective effects on endothelial function. Aim of this study was to evaluate HDL function in patients with chronic heart failure (CHF), the impact of ET and the respective molecular pathways.

Methods: HDL was isolated from 8 healthy controls ($HDL_{healthy}$) before and after ET, , 8 patients with CHF-NYHA-II ($HDL_{NYHA-II}$), and 8 patients with CHF-NYHA-IIIB ($HDL_{NYHA-IIIb}$) before and after ET. Endothelial cells (EC) were incubated with HDL and phosphorylation of eNOS-Ser^{1177}, eNOS-Thr^{495}, PKC-ßII-Ser^{660} and p70S6K-Ser^{411} was evaluated. HDL-bound malondialdehyde and HDL-induced NO production by EC was quantified. The proteome of HDL particles were investigated by shotgun LC-MS/MS. Endothelial function was assessed by flow-mediated dilatation (FMD).

Results: Incubation of ECs with $HDL_{NYHA-IIIb}$ reduced phosphorylation of eNOS-Ser^{1177}, whereas phosphorylation at eNOS-Thr^{495} increased when compared to $HDL_{healthy}$. This was associated with lower NO production of ECs incubated with $HDL_{NYHA-IIIb}$. In addition an elevated activation of p70S6K, PKC-ßII by $HDL_{NYHA-IIIb}$, and a higher amount of malondialdehyde bound to $HDL_{NYHA-IIIb}$ was measured.

ET of healthy individuals had no effect on HDL function, wheres ET of CHF-NYHA-IIIB significantly improved HDL function. A correlation between changes in HDL-induced NO production and FMD improvement by ET was evident.

Discussion: These results demonstrate that HDL-function is significantly impaired in CHF and that ET improved the vascular effects of HDL. This may be one mechanism how ET exerts its beneficial effects in CHF.

Introduction

An abnormality in endothelium-dependent vasodilatation is a key phenomenon in patients with chronic heart failure (CHF). Numerous animal [1,2] and human [3,4] studies demonstrated a significant improvement in endothelium-dependent relaxation by exercise training (ET). An important factor responsible for this improvement is the increase in the bioavailability of nitric oxide (NO). Studies using cultured endothelial cells [5,6] or vessel tissue specimens obtained from trained animals [7] or humans [8] clearly documented that an increase in shear stress resulted in activation of endothelial nitric oxide synthase (eNOS), either by increased expression [9] or an activation by phosphorylation [8], and a reduced production of reactive oxygen species competing for NO [10].

High-density lipoprotein (HDL) levels above 40-60 mg/dl have been proposed as strong independent predictor of lower coronary artery disease risk [11,12,13]. Besides promotion of reverse cholesterol transport, HDL has been found to exert important anti-atherogenic effects by stimulation of endothelial cell NO production and endothelial repair as well as anti-inflammatory and anti-oxidant effects [14,15,16,17,18,19]. In recent studies it became evident that the functional properties of HDL with respect to stimulation of NO production is significantly impaired in patients with diabetes [20], coronary artery disease [21], and primary antiphospholipid syndrome [22]. Mechanistically it is proposed that in these patients malondialdehyde-modified HDL triggers the activation of PKC-ßII, thereby reducing eNOS-dependent NO production [21]. Based on these findings on the functional importance of HDL in influencing endothelial function, the therapeutic approach targeting HDL is beginning to shift towards improving HDL function rather than just increasing its concentration (reviewed in [23]).This view is further supported by the recently published dal-OUTCOMES trail, documenting that just raising the HDL level does not reduce the risk of recurrent cardiovascular events [24].

To critically assess the relation between disease severity of patients with CHF and HDL

function, we isolated HDL from healthy, CHF patients in NYHA class II and IIIB and determined its ability to stimulate eNOS activation and thereby NO production. To study the impact of ET on HDL function, HDL was additionally isolated from healthy individuals and NYHA-IIIb patients after an ET program and its stimulating effect on eNOS phosphorylation and NO production was evaluated.

Methods

Patient population and blood sampling

Eight healthy control subjects (HS) with normal systolic LV function (>55%), without signs or symptoms of CHF and without evidence of coronary artery disease during coronary angiography were included into the study. The HS performed an ET program as outlined below for 4 weeks. In addition 8 patients with CHF (LVEF <40%) in NYHA class II and 8 CHF patients in NYHA class IIIb were recruited. Patients in NYHA IIIb were assigned to a 12 week ET program as outlined below.

Blood was collected from all subjects when entering the study and when finishing the ET program (only healthy and NYHA IIIb subjects). Serum was prepared by ultracentrifugation (10 min at 3000 x g at 4°C) and stored at -80°C until used.

Training protocol of patients in NYHA IIIb

The initial phase of the exercise program was supervised and performed in-hospital. During the first 3 weeks, patients exercised 3 to 6 times daily for 5 to 20 minutes on a bicycle ergometer adjusted to the work load at which 50% of maximum oxygen uptake (VO_2max) was reached. Before discharge from the hospital, symptom-limited spiroergometry was performed again to determine the training target heart rate for home

based training (defined as the heart rate reached at 60% of VO_2max). On discharge, patients were provided with bicycle ergometers for home based ET. They were encouraged to exercise close to their target heart rate for 20 to 30 minutes daily for a period of 12 weeks and were expected to participate in 1 supervised group training session for 60 minutes every week consisting of walking, calisthenics, and noncompetitive ball games.

Measurement of endothelial function

Flow-mediated dilatation (FMD) of the radial artery was measured using a high-resolution ultrasound scanning echo-tracking angiometer (NIUS 02, Asulab Research Laboratory, Neuchatel, Switzerland) as described previously [25,26].

Isolation of HDL

HDL was isolated from serum by sequential density ultracentrifugation (d = 1.006 – 1.21 g/ml) as recently described in detail. [20,21] Quality of isolated HDL was evaluated by polyacrylamid gel electrophoresis followed by coomassie blue staining.

Cell culture and incubation with isolated HDL

Human aortic endothelial cells (HAEC, Cell Systems Biotechnology, Troisdorf, Germany) were cultured in EGM-2 cell culture medium (Lonza, Wlkersville MD, USA) until 80 to 90% confluence. Cells were incubated for 0, 5, 10, 15, 30 or 60 min with 50 µg/ml isolated HDL. To elucidate signaling pathways cells were pretreated (1h prior HDL stimulation) with specific inhibitors like rapamycin (Santa Cruz, Heidelberg, Germany; 20 nmol/l) to inhibit p70S6K or CG53353 (Merck Chemicals, Nottingham, UK; 2 µmol/L) to inhibit PKC-ßII. Thereafter, cells were harvested with ice cold lysis buffer (50 mmol/L Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Triton X-100,

0.2% SDS) containing protease inhibitor mix M (Serva, Heidelberg, Germany) as well as phosphatase inhibitors (Phosphatase inhibitor mix II, Serva, Heidelberg, Germany). Protein concentration was determined using BSA as standard (BCA method, Pierce, Rockford IL, USA).

Western blot analysis

Ten µg of total protein was separated on a denaturing polyacrylamid gel and transferred to a PVDF membrane. To detect specific proteins the following antibodies were applied: anti-endothelial nitric oxide synthase (eNOS) (Santa Cruz, Heidelberg, Germany), anti phospho-eNOS-Ser^{1177}, anti phospho-eNOS-Thr^{495} (both BD Bioscience, Heidelberg, Germany), anti-PKC-ßII, anti-phospho PKC-ßII-Ser^{660}, anti-p70S6Kα, anti-phospho p70S6Kα-Ser^{411} (all Santa Cruz, Heidelberg, Germany). All samples were analyzed in triplicate.

Measurement of endothelial cell NO production by ESR spectroscopy

HAEC cell were incubated with HDL (50 µg/ml) for 60 min. Nitric oxide production in human aortic endothelial cells (HAECs) was measured by ESR spectroscopy using the spin-probe colloid $Fe(DETC)_2$ as recently described [20,21].

Measurement of Pon1 enzymatic activity

Paraoxonase activity of HDL-associated paraoxonase-1 (PON1) was measured by spectrophotometry using paraoxon as substrate. [21]

Quantification of protein-bound malondialdehyd in HDL

Free and protein-bound malondialdehyde (MDA) in HDL was detected by a commercially available lipid peroxidation assay kit (ALDetect, Enzo Life Sciences) as recently described.

Measurement of plasma oxidative capacity

Oxidative capacity was determined in plasma samples from all participants using a commercial available quantification kit (PerOx (KC5100) kit, Immundiagnostik, Bensheim, Germany). Measurements were performed in duplicate according to the manufactures recommendations.

NanoLC-MS/MS analysis

The proteome of isolated HDL particles were investigated by shotgun LC-MS/MS (for detailed description see online supplement).

Statistical Analysis

SPSS version 16.0 (SPSS Inc, Chicago, Ill) was used for all of the analyses. Data are expressed as mean±SEM. Comparisons among groups were tested with ANOVA. When data were not normally distributed or the variance was not equal, the Kruskal-Wallis nonparametric test was used. A value of $p<0.05$ was considered statistically significant. All of the measurements were made by investigators blinded to the treatment group.

Results

Patient characteristics and follow-up after exercise training

The baseline values for all individuals included into the study are depicted in Table 1.

As expected the healthy individuals significantly differed from patients with chronic heart failure with respect to LV ejection fraction, maximal oxygen consumption and medication. No difference was observed between the three groups comparing age, BMI, arterial hypertension and diabetes mellitus. None of the participants was active smoking. Analyzing the lipid profile, only a significant lower HDL concentration was evident in CHF patients in NYHA class IIIb when compared to healthy individuals.

The exercise program of 12 weeks in NYHA class IIIb patients led to an increase of VO_2max (14.3±1.2 to 17.4±1.6 ml/kg/min; $p<0.05$) and left ventricular ejections fraction (24±2 to 34±2 %; $p<0.05$). With respect to the lipid profile no change after 12 weeks of ET was evident.

HDL-mediated eNOS phosphorylation and NO production – impact of exercise training

Incubating HAEC with $HDL_{healthy}$ the phosphorylation of eNOS at position Ser^{1177} was increased 5.0±0.5 fold vs. unstimulated cells. This stimulation of eNOS-Ser^{1177} was lower with $HDL_{NYHA-II}$ (3.7±1.1 fold vs. unstimulated cells; p=NS vs. healthy controls), reaching significance in CHF patients in NYHA class IIIb (1.8±0.4 fold vs. unstimulated cells; $p<0.05$ vs. healthy controls) (Figure 1A). Analyzing the eNOS phosphorylation at position Thr^{495}, $HDL_{NYHA-IIIb}$ stimulated the phosphorylation significantly more than $HDL_{healthy}$ (NYHA IIIb: 3.3±0.4 fold vs. unstimulated cells; NYHA II: 2.6±0.5 fold vs. unstimulated cells; healthy controls: 1.5±0.4 fold vs. unstimulated cells; $p<0.05$ NYHA IIIb vs. healthy controls). (Figure 1B). These differences in HDL-mediated eNOS phosphorylation capacity between the groups are also evident when measuring the HDL-induced NO production of

endothelial cells (Figure 2).

Comparing the functional properties of $HDL_{NYHA\text{-}IIIb}$ before and after an exercise program revealed a significant improvement in phosphorylation of eNOS at position Ser^{1177} (begin: 1.8±0.4 fold vs. unstimulated cells; end: 4.4±0.9 fold vs. unstimulated cells; $p<0.05$) (Figure 1A), a significant reduction in phosphorylating eNOS at position Thr^{495} (begin: 3.3±1.0 fold vs. unstimulated cells; end: 2.1±0.4 fold vs. unstimulated cells; $p<0.05$) (Figure 1B), and a significant higher production of NO in endothelial cells (begin: -1.0±3.3 % increase versus buffer treated cells; end: 19.4±7.5 % increase versus buffer treated cells; $p<0.01$) (Figure 2). No significant impact of exercise training was observed in the healthy control group with respect to HDL-mediated eNOS-$Serr^{1177}$ phosphorylation (begin: 5.0±0.5 fold vs. unstimulated cells; end: 4.0±1.0 fold vs. unstimulated cells; p=NS), eNOS-Thr^{495} phosphorylation (begin: 1.5±0.4 fold vs. unstimulated cells; end: 1.5±0.3 fold vs. unstimulated cells; p=NS) (Figure 1A and B) and NO production (Figure 2).

HDL-mediated PKC-ßIISer^{660} phosphorylation

Incubation of HAEC with $HDL_{healthy}$ resulted in a 1.4±0.3 fold increase in PKC-ßII phosphorylation at position Ser^{660} when compared to unstimulated cells. This ability to stimulated PKC-ßII phosphorylation is increased with increasing severity of CHF (NYHA II: 1.8±1.1 fold vs. unstimulated cells, NYHA IIIb: 2.2±0.7 fold vs. unstimulated cells; $p<0.05$ NYHA IIIb vs. healthy) (Figure 3A). Performing an exercise program in patients with CHF NYHA class IIIb significantly reduced the ability of HDL to phosphorylate PKC-ßII (begin: 2.2±0.7 vs. end: 1.4±0.2 fold vs. unstimulated cells; $p<0.05$) (Figure 3A). No impact of ET on HDL-induced phosphorylation of PKC-ßII was observed in the healthy controls (begin: 1.4 ±0.3 vs. end: 1.6±0.2 fold vs. unstimulated cells; p=NS) (Figure 3A).

To evaluate the importance of PKC-ßII activation for HDL-induced eNOS-phosphorylation, the $HDL_{NYHA\text{-}IIIb}$ induced eNOS phosphorylation at Ser^{1177} and Thr^{495} was measured in the

absence or presence of a specific PKC-ßII inhibitor (CG53353). As shown in Figure 3B the inhibition of PKC-ßII prevented the HDL-induced phosphorylation at eNOS-Thr^{495} without having any influence on eNOS-Ser^{1177} phosphorylation.

HDL-mediated p70S6K-Ser^{411} phosphorylation

Incubation of HAEC with $HDL_{healthy}$ resulted in a 1.5±0.2 fold increase in p70S6K phosphorylation at position Ser^{411} when compared to unstimulated cells. This ability to stimulated p70S6K phosphorylation is increased with increasing severity of CHF (NYHA II: 1.9±0.4 fold vs. unstimulated cells, NYHA IIIb: 3.1±0.7 fold vs. unstimulated cells; $p<0.05$ NYHA IIIb vs. healthy) (Figure 4A). Performing an exercise program in patients with CHF NYHA class IIIb significantly reduced the ability of HDL to phosphorylate PKC-ßII (begin: 3.1±0.7 vs. end: 1.4±0.1 fold vs. unstimulated cells; $p<0.01$) (Figure 4A). No impact of ET on HDL-induced phosphorylation of PKC-ßII was observed in the healthy controls (begin: 1.5 ±0.2 vs. end: 1.7±0.2 fold vs. unstimulated cells; p=NS) (Figure 4A).

Blocking PKC-ßII activation by CG53353 resulted in an inhibition of $HDL_{NYHA-IIIb}$-induced phosphorylation of p70S6K at Ser^{411} (Figure 4B). In addition, blocking p70S6K activation by rapamycin prevented the $HDL_{NYHA-IIIb}$-induced phosphorylation of eNOS at Thr^{495} without influencing eNOS phosphorylation at Ser^{1177} (Figure 4 C/D).

Malondialdehyde bound to HDL

HDL-bound MDA was significantly increase in $HDL_{NYHA-IIIb}$ when compared to $HDL_{healthy}$ (healthy: 0.39±0.09 nmol/mg HDL; NYHA II: 0.64±0.09 nmol/mg HDL; NYHA IIIb: 1.13±0.10 nmol/mg HDL; $p<0.001$ healthy vs. NYHA IIIb, $p<0.05$ NYHA II vs. NYHA IIIb) (Figure 5A). After finishing an ET program the amount of HDL-bound MDA was significantly reduced by 27.4±6.1% in CHF-NYHA-IIIb, whereas no change was observed in healthy controls (Figure 5B).

Pon-1 enzyme activity and total lipid peroxides

Measuring the paraoxonase enzymatic activity of PON-1 associated with the isolated HDL, a significant decrease in $HDL_{NYHA\text{-}IIIb}$ was evident when compared to $HDL_{healthy}$ (Figure 5C). It also became evident, that in the patients in NYHA IIIb assigned to an exercise intervention program no change in paraoxonase (Figure 5C).

Evaluating circulating total lipid peroxides (LPO) as measure for the oxidative status, a significant increase was obvious in the plasma of CHF NYHA IIIb patients when compared to healthy controls (healthy: 151±41 vs. NYHA-IIIb: 351±71 µmol/L; $p<0.05$). Furthermore, the ET intervention let to a significant reduction of LPO (Begin NYHA-IIIb: 351±71 vs. end: NYHA-IIIb: 210±54 µmol/L; $p<0.05$) (Figure 5D). No impact of ET on LPO was evident in healthy controls (Figure 5D).

Correlation between HDL and endothelial function

To investigate if the exercise-induced change in HDL function observed in CHF NYHA IIIb patients has some influence on endothelial function, a correlation analysis between the change in HDL function and the observed change in endothelial function was performed. A significant correlation was evident between the change in HDL-induced eNOS-Ser^{1177} phosphorylation ($r=0.81$, $p<0.05$; see supplementary Figure 1A) or the absolute change in HDL-induced NO production ($r=0.78$, $p<0.05$; see supplementary Figure 1B) and the change in endothelial function.

Proteome analysis of HDL particles

In the complete LC-MS/MS dataset, 134 proteins from 709 distinct peptides were identified using the Mascot algorithm; 65 proteins with at least one unique peptide per protein and 69 proteins with at least two peptides per protein were identified.

A Venn's four-set diagram using ellipses shows common and unique proteins between the analyzed groups (Figure 6A). Only a very small proportion of proteins were identified uniquely for one treatment group. The proteins identified in all groups (n=75) were further used for direct label free quantitation utilizing peptide intensities as indicator for protein abundance. A principal component analysis (PCA) plot as indicator of the variance in the dataset (n=134 proteins) is shown in Figure 6B. Each point represents one LC-MS/MS run of one subject. The plot displays the first two principal components that accounts for about 30% of the global variance. A clear distinction of the HDL proteome was observed between the healthy controls (pre and post intervention) and CHF-NYHA-IIIb patients (pre and post intervention). However ET did not result in a clear separation of the HDL proteome in healthy controls (Beg. Vs. end) and CHF-NYHA-IIIb patients (Beg. vs. end; Figure 6B).

To define significantly abundant proteins between healthy controls and CHF-NYHA-IIIb patients a independent T-test as statistical analysis was applied. A Volcano plot shows the significance versus effect size-change on the y- and x-axes, respectively to quickly visualize the most meaningful changes (Figure 6C/D). The comparison of controls and NYHA patients at study begin (Figure 6C) indicated 13 statistically relevant proteins shown as red marked spots. After intervention, 17 proteins were differentially regulated between controls and NYHA patients (Figure 6D, Table 1). A dependent (paired) T-Test was performed to identify significant relevant proteins within the control and the patients groups influenced by ET. However, only one protein (Apolioprotein C-II (APOC2) with a minor effect size (effect size of 0.19 is means 1.15 fold change) was statistically differential in the healthy control group (Table 1). Additionally, we identified protein groups that were not previously identified in HDL particles, such as beta-Ala-His dipeptidase (CNDP1), pulmonary surfactant-associated protein B (SFTPB) or leucine-rich repeat-containing protein 15 (LRRC15)

Discussion

HDL shows many anti-atherosclerotic properties, including reverse cholesterol transport, decreasing oxidation of LDL and the expression of adhesion molecules (for review see [19]), stimulating eNOS-dependent NO production [14,27,28] and mediation of endothelial repair mechanisms [29,30,31]. In recent years it became evident that not only the quantity but also the functional capacity of HDL is important for influencing the risk of cardiovascular disease [32]. Therefore, strategies are developed to increase HDL quantity and function [23]. ET is an accepted intervention strategy in patients with systolic heart failure [33] and part of the molecular mechanisms behind the beneficial effect of ET have been described (for a detailed review see [34]). With respect to functional properties of HDL in patients with chronic heart failure and the impact of ET several findings emerge from the present study. First, the functional capacity of HDL to increase NO production in endothelial cells via modulation of eNOS phosphorylation is impaired in patients with chronic heart failure. This functional incompetence is gradually increasing with disease severity. Second, in CHF patients the amount of MDA bound to HDL is significantly elevated and the activation of PKC-ßII/p70S6K by $HDL_{NYHA\text{-}IIIb}$ is significantly increased when compared to $HDL_{healthy}$. Third, ET in CHF patients in NYHA IIIb for 12 weeks significantly restored the functional capacity of HDL as well as the amount of MDA bound to HDL and the HDL-mediated activation of PKC-ßII/p70S6K. The ET-induced change in HDL function significantly correlates with an improved endothelial function. Fourth, proteom analysis of the HDL particle revealed a clear distinction between HDL isolated from healthy controls and CHF – NYHA-IIIb patients. No significant impact of ET on the overall composition of the HDL particle was evident.

These findings suggest, that ET has a positive effect on HDL function with respect to NO generation, and this might be one pathway how ET improves endothelial function in

patients with CHF.

Chronic heart failure and the ability of HDL to regulate NO production

Experimental and clinical studies have suggested that eNOS-derived NO is a crucial determinant of vascular homeostasis, and reduced bioavailability of NO plays an important role in the development and progression of atherosclerosis [35]. Especially in patients with coronary artery disease the amount of bioavailable NO is significantly reduced due to lower expression of eNOS [8] or an increased concentration of reactive oxygen species (ROS) [10]. But also patients with chronic heart failure irrespective of atherosclerotic etiology are characterized by endothelial dysfunction as a result of increased oxidative stress and reduced NO bioavailability [36].

The activity of eNOS is regulated by intracellular calcium concentration, by modulating the protein concentration per se or by phosphorylation at specific residues via activation of protein kinase B (Akt) or protein kinase A (PKA) (for detailed review see [37]). Besides the activation of eNOS by shear stress or agonists like acetylcholine, bradykinin, or vascular endothelial growth factor (VEGF), HDL also has the potency to activate eNOS by binding to the scavenger receptor-B1 [14]. In the present study we described for the first time that the capacity of HDL isolated from CHF patients to phosphorylate eNOS and generate NO is significantly impaired. This reduced ability of HDL to stimulate eNOS activity (increased phosphorylation at position Thr^{495} and reduced phosphorylation at position Ser^{1177}) even seems to be related to disease severity, since the most relevant impairment was seen in CHF patients in NYHA class IIIb followed by patients in NYHA II when compared to healthy controls. This loss in functional capacity goes along with a significant reduction in NO. This result is in good agreement with findings in other atherosclerotic disorders like primary antiphospholipid syndrome [22], diabetes [20], and patients with stable CAD or acute coronary syndrome [21]. This functional reduction of HDL is on top of a quantitative reduction of HDL

which was noted in the patients described in the present study and in the current literature [38], resulting in a lower anti-atherosclerotic effect of HDL.

Impact of exercise training on HDL function

ET has been proven to partially correct endothelial dysfunction in a variety of diseases (for review see [39]). As molecular mechanisms restoration of NO bioavailability due to an increase in eNOS expression and Akt-mediated phosphorylation of eNOS at position Ser^{1177} and the reduction in ROS are discussed [8, 10]. Using cultured human endothelial cells and $HDL_{NYHA\text{-}IIIb}$ isolated before and after an ET intervention program, we could clearly demonstrate an improvement in HDL function, measured as the capacity to phosphorylate eNOS at Ser^{1177} and Thr^{495}, culminating in an increased NO production. The positive effect of ET on HDL function is in line with a study performed on overweight/obese men with cardiovascular risk factors [40]. In that study a 21-day intervention program consisting of high-fiber low fat diet combined with an exercise program (daily treadmill walking, 45-60 min, 70-85% of maximal heart rate) resulted in a lower HDL inflammatory index. Unfortunately the authors did not analyze the individual effects, so it is not possible to differentiate which intervention, diet or ET, is responsible for the observed effect. Based on the results presented in this study one may speculate that the training induced increase in NO bioavailability and finally the improvement of endothelial function is partly due to an altered HDL function. This assumption is at least supported by the positive correlation between the exercise-induced change in HDL function (change in HDL mediated phosphorylation of eNOS or HDL-mediated NO production) and the change in endothelial function. This functional improvement of HDL by ET is realized without a change in total HDL concentration. This finding highlights to an important point, namely that the functional competence of HDL may be more important for regulating endothelial function/antitrombotic function than the absolute amount.

Possible molecular mechanisms

As already described for HDL isolated from patients with stable coronary artery disease or an acute coronary syndrome [21], HDL obtained from NYHA IIIb patients significantly activated PKC-ßII and subsequently p70S6K via phosphorylation at Ser^{660} or Ser^{411} respectively . Inhibition studies using either CG53353 or rapamycin clearly documented that the activation of p70S6K is downstream of PKC-ßII activation, and that the HDL mediated activation of this pathways influences mainly the phosphorylation of eNOS at the inhibitory site Thr^{495}. Of note, this activation of the PKC-ßII pathway was significantly reduced in CHF-patients after an ET program. In addition, using either transgenic mice or endothelial cell culture it could be documented that activation of PKC-ßII in endothelial cells and vascular tissue inhibits Akt-dependent regulation of eNOS [41,42]. A central question based on this observation is what discriminates HDL isolated from healthy controls and patients with CHF in NYHA IIIb in its ability to activate PKC-ßII/p70S6K? At least two possibilities should be discussed. First, a change in the overall protein composition of the HDL particle and second a secondary modification of the HDL particle. With respect to protein composition a clear distinction was evident between the healthy controls and CHF-NYHA-IIIb patients. However ET did not result in a significant modification in the HDL proteome, despite an improved HDL-mediated NO production. Second, an other potential mechanism would be the modification of apolipoprotein A-1, the major HDL protein, by reactive intermediates. Indeed impaired HDL-function after modification by MDA or myristic acid could be documented [43,44,45]. For example MDA modification of HDL decreased cholesterol efflux from cultured human fibroblasts [43] or rat liver endothelial cells [44]. Notably, in the present study we have observed that a significant higher level of MDA is bound to $HDL_{NYHA\text{-}IIIb}$ in comparison to $HDL_{healthy}$ and that a training intervention significantly reduced

the MDA amount bound to $HDL_{NYHA-IIIb}$. Supported by in vitro studies [21] it is reasonable to assume that the amount of MDA bound to HDL regulates the activation of PKC-ßII (higher activation of PKC-ßII in case more MDA is bound to HDL) and finally the activation of eNOS and NO generation. Since the modulation of HDL by MDA seems to play such a central role, the question arises which factors are regulating the amount of MDA bound to HDL, and are they influenced by ET? Paraoxonase (Pon), an enzyme which is associated with HDL, protects lipoproteins (HDL and LDL) from oxidative modification. It is speculated that a lower level of Pon activity in the serum is associated with an increased risk of atherosclerosis. Indeed, in obese subjects with a higher risk for cardiovascular disease, a lower Pon activity was measured [46]. This relation between Pon activity and the risk for cardiovascular disease was confirm in recent clinical studies [47,48]. In the present study Pon activity of $HDL_{NYHA-IIIb}$ was significantly decreased when compared to $HDL_{healthy}$. Unfortunately, ET had no effect on Pon activity. This is in accordance with a study performed in obese men, showing improved HDL inflammatory/anti-inflammatory properties after a lifestyle intervention (combination of diet and an exercise intervention) without changing Pon activity [40]. Therefore, at the moment it remains unclear which mechanism beside Pon may be responsible for the ET-mediated reduction in MDA bound to HDL. Of note an influence of ET on the total oxidative capacity, measured as lipid peroxides in the patient plasma, was evident. Therefore one may speculate that ET reduces the oxidative load, and thereby reduced the amount of MDA bound to HDL.

Study limitations

Some limitations of the present study should be mentioned.

First, only 8 patients or healthy individuals were included into each group. Nevertheless, the primary study goal was to investigate if HDL function in patients with CHF is altered and if this can be reversed by ET. Even with such a small number of individuals in each

group a significant impaired HDL function in NYHA IIIb was evident which could be improved by ET. In addition no data are available on the impact of ET in CHF-NYHA-class II patients.

Second, it remains unclear which mechanism is responsible for the exercise-induced reduction of MDA bound to HDL. According to the data presented in this study the activity of the paraoxogenase is not altered by ET, and therefore can be excluded. On the other hand we could observe an exercise-induced reduction in lipid peroxidation which is a general measure for the oxidative load. So far, it remains unclear which enzymatic system is responsible for the observed reduction in oxidative stress.

Third, at the moment we do not know, if the alterations in the HDL-proteome observed between $HDL_{healthy}$ and $HDL_{NYHA\text{-}IIIb}$ have any causal relevance for HDL-mediated eNOS phosphorylation. To answer this questions further studies using site directed mutagenesis are warranted.

Fourth, the results presented in the study are obtained in cell culture experiments using HDL isolated from frozen serum of different patient cohorts. Therefore, it has to be answered if this modulation of HDL also has an impact on endothelial function in vivo. At least the correlation detected between the improvement in endothelial function induced by ET measured in vivo and the HDL associated change in eNOS phosphorylation and NO generation measured in vitro implies that this is an important mechanism for the regulation of endothelial function in vivo. With respect to storage of the serum at -80°C and functionality, no data with respect to HDL-mediated eNOS phosphorylation are available. Nevertheless, in a recent study investigating the HDL-mediated cholesterol efflux, no difference was seen between HDL isolated from fresh serum or serum stored for up to two years [49].

Conclusion

In summary, the reduced ability of HDL to stimulate endothelial NO production via activation of eNOS in patients with advanced chronic heart failure suggest a loss of this functional property of HDL. Of note an ET program in this patient cohort seems to partially correct this dysfunction. This may be one possible explanation for the beneficial effect of ET on endothelial function. The exercise-mediated effect on HDL function seems to be mediated via a reduction in MDA bound to HDL, leading finally to a lower activation of PKC-ßII resulting in a higher activation of eNOS and a higher synthesis of NO (Figure 7).

Acknowledgements

We would like to thank Dr. Andreas Schubert, Fraunhofer Institute, Leipzig, Germany for providing the access to an ultracentrifuge for HDL isolation.

Source of Funding

The study was supported by a grant from the German Heart Foundation (Dr. Erbs). The study was supported by a grant from the Leducq Foundation and from the Swiss National Foundation (138486).

Disclosures

None.

References

1. Kemi OJ, Haram PM, Loennechen JP, Osnes JB, Skomedal T, Wisloff U, Ellingsen O. Moderate vs. high exercise intensity: Differential effects on aerobic fitness, cardiomyocyte contractility, and endothelial function. Cardiovasc Res 2005;67:161-172.

2. Haram PM, Adams V, Kemi OJ, Brubakk AO, Hambrecht R, Ellingsen O, Wisloff U. Time-course of endothelial adaptation following acute and regular exercise. Eur J Cardiovasc Prev Rehabil 2006;13:585-591.

3. Hambrecht R, Fiehn E, Weigl C, Gielen S, Hamann C, Kaiser R, Yu J, Adams V, Niebauer J, Schuler G. Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. Circulation 1998;98:2709-2715.

4. Hambrecht R, Wolff A, Gielen S, Linke A, Hofer J, Erbs S, Schoene N, Schuler G. Effect of exercise on coronary endothelial function in patients with coronary artery disease. New England Journal of Medicine 2000;342:454-460.

5. Dimmeler S, Hermann C, Galle J, Zeiher AM. Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. Arterioscler Thromb Vasc Biol 1999;19:656-664.

6. Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, Jo H. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. J Biol Chem 2002;277:3388-3396.

7. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349-353.

8. Hambrecht R, Adams V, Erbs S, Linke A, Kränkel N, Shu Y, Baither Y, Gielen S, Gummert JF, Mohr FW, Schuler G. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. Circulation 2003;107:3152-3158.

9. Tanabe T, Maeda S, Miyauchi T, Iemitsu M, Takanashi M, Irukayama-Tomobe Y, Yokota T, Ohmori H, Matsuda M. Exercise training improves aging-induced decrease in eNOS expression of the aorta. Acta Physiol Scand 2003;178:3-10.

10. Adams V, Linke A, Kränkel N, Erbs S, Gielen S, Möbius-Winkler S, Gummert JF, Mohr FW, Schuler G, Hambrecht R. Impact of regular physical activity on the NAD(P)H oxidase and angiotensin receptor system in patients with coronary artery disease. Circulation 2005;111:555-562.

11. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 1977;62:707-714.

12. Assmann G, Schulte H, von Eckardstein A, Huang Y. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. Atherosclerosis 1996;124, Supplement:S11-S20.

13. Di Angelantonio E, sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J. Major lipids,apolipoproteins, and risk of vascular disease. JAMA 2009;302:1993-2000.

14. Yuhanna IS, Zhu Y, Cox BE, Hahner LD, Osborne-Lawrence S, Lu P, Marcel YL, Anderson RG, Mendelsohn ME, Hobbs HH, Shaul PW. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. Nat Med 2001;7:853-857.

15. Yvan-Charvet L, Wang N, Tall AR. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. Arterioscler Thromb Vasc Biol 2010;30:139-143.

16. Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, Wang N. HDL, ABC transporters, and cholesterol efflux: Implications for the treatment of atherosclerosis. Cell Metab 2008;7:365-375.

17. Rye KA, Bursill CA, Lambert G, Tabet F, Barter PJ. The metabolism and anti-atherogenic properties of HDL. J Lipid Res 2009;50:S195-S200.

18. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. Circ Res 2004;95:764-772.

19. Navab M, Reddy ST, Van Lenten BJ, Fogelman AM. HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. Nat Rev Cardiol 2011;8:222-232.

20. Sorrentino SA, Besler C, Rohrer L, Meyer M, Heinrich K, Bahlmann FH, Mueller M, Horvath T, Doerries C, Heinemann M, Flemmer S, Markowski A, Manes C, Bahr MJ, Haller H, von Eckardstein A, Drexler H, Landmesser U. Endothelial-vasoprotective effects of high-density lipoprotein are impaired in patients with type 2 diabetes mellitus but are improved after extended-release Niacin therapy. Circulation 2010;121:110-122.

21. Besler C, Heinrich K, Rohrer L, Doerries C, Riwanto M, Shih DM, Chroni A, Yonekawa K, Stein S, Schaefer N, Mueller M, Akhmedov A, Daniil G, Manes C, Templin C, Wyss C, Maier W, Tanner FC, Matter CM, Corti R, Furlong C, Lusis AJ, von Eckardstein A, Fogelman AM, Lüscher TF, Landmesser U. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. J Clin Invest 2011;121:2693-2708.

22. Charakida M, Besler C, Batuca JR, Sangle S, Marques S, Sousa M, Wang G, Tousoulis D, gado Alves J, Loukogeorgakis SP, Mackworth-Young C, D´Cruz D, Luscher T, Landmesser U, Deanfield JE. Vascular abnormalities, paraoxonase activity, and dysfunctional HDL in primary antiphospholipid syndrome. JAMA 2009;302:1210-1217.

23. Duffy D, Rader DJ. Update on strategies to increase HDL quantity and function. Nat Rev Cardiol 2009;6:455-463.

24. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, Chaitman BR, Holme IM, Kallend D, Leiter LA, Leitersdorf E, McMurray JJV, Mundl H, Nicholls SJ, Shah PK, Tardif JC, Wright RS. Effects of Dalcetrapib in Patients with a Recent Acute Coronary Syndrome. New England Journal of Medicine 2012;Nov 5. [Epub ahead of print].

Formatiert: Norwegisch (Bokmål)

25. Erbs S, Höllriegel R, Linke A, Beck EB, Adams V, Gielen S, Möbius-Winkler S, Sandri M, Kränkel N, Hambrecht R, Schuler G. Exercise training in patients with advanced chronic heart failure (NYHAIIIb) promotes restoration of peripheral vasomotor function, induction of endogenous regeneration, and improvement of left ventricular function. Circ Heart Fail 2010;3:486-494.

26. Linke A, Schoene N, Gielen S, Hofer J, Erbs S, Schuler G, Hambrecht R. Endothelial dysfunction in patients with chronic heart failure: systemic effects of lower-limb exercise training. J Am Coll Cardiol 2001;37:392-397.

27. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. J Biol Chem 2003;278:9142-9149.

28. Nofer JR, Van der Giet M, Tölle M, Wolisnka I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Gödecke A, Ishii I, Kleuser B, Schäfers M, Fobker M, Zidek w, Assmann G, Chun J, Levkau B. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor $S1P_3$. J Clin Invest 2004;113:569-581.

29. Tsa C, Martinic G, Fan WH, Rogers C, Rye KA, Barter PJ. High-density lipoproteins enhance progenitor-mediated endothelium repair in mice. Arterioscler Thromb Vasc Biol 2006;26:1144-1149.

30. Noor R, Shuaib U, Wang CX, Todd K, Ghani U, Schwindt B, Shuaib A. High-density lipoprotein cholesterol regulates endothelial progenitor cells by increasing eNOS and preventing apoptosis. Atherosclerosis 2007;192:92-99.

31. Petoumenos V, Nickenig G, Werner N. High-density lipoprotein exerts vasculoprotection via endothelial progenitor cells. J Cell Mol Med 2009;13:4623-4635.

32. Gielen S, Landmesser U. A new look at HDL in coronary disease: can we escape natural history? Heart 2011;97:1899-1901.

33. Downing J, Balady GJ. The role of exercise training in heart failure. J Am Coll Cardiol 2011;58:561-569.

34. Gielen S, Schuler G, Adams V. Cardiovascular effects of exercise training: Molecular mechanisms. Circulation 2010;122:1221-1238.

35. Landmesser U, Hornig B, Drexler H. Endothelial function: A critical determinant in atherosclerosis? Circulation 2004;109:II-27-II-33.

36. Erbs S, Beck EB, Linke A, Adams V, Gielen S, Kränkel N, Möbius-Winkler S, Höllriegel R, Thiele H, Hambrecht R, Schuler G. High-dose rosuvastatin in chronic heart failure promotes vasculogenesis, corrects endothelial function, and improves cardiac remodeling: Results from a randomized, double-blind, and placebo-controlled study. Int J Cardiol 2011;146:56-63.

37. Kolluru GK, Siamwala JH, Chatterjee S. eNOS phosphorylation in health and disease. Biochimie 2010;92:1186-1198.

38. Rauchhaus M, Koloczek V, Volk H, Kemp M, Niebauer J, Francis DP, Coats AJ, Anker SD. Inflammatory cytokines and the possible immunological role for lipoproteins in

chronic heart failure. Int J Cardiol 2000;76:125-133.

39. Linke A, Erbs S, Hambrecht R. Effects of exercise training upon endothelial function in patients with cardiovascular disease. Front Biosci 2008;13:424-432.

40. Roberts CK, Ng C, Hama S, Eliseo AJ, Barnard RJ. Effect of a short-term diet and exercise intervention on inflammatory/anti-inflammatory properties of HDL in overweight/obese men with cardiovascular risk factors. J Appl Physiol 2006;101:1727-1732.

41. Naruse K, Rask-Madsen C, Takahara N, Ha SW, Suzuma K, Way KJ, Jacobs JRC, Clermont AC, Ueki K, Ohshiro Y, Zhang J, Goldfine AB, King GL. Activation of vascular protein kinase C-ß inhibits Akt-dependent endothelial nitric oxide synthase function in obesity-associated insulin resistance. Diabetes 2006;55:691-698.

42. Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, Feener EP, Herbert TP, Rhodes CJ, King GL. Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo : A specific vascular action of insulin. Circulation 2000;101:676-681.

43. Salmon S, Mazière C, Auclair M, Theron L, Santus R, Mazière JC. Malondialdehyde modification and copper-induced autooxidation of high-density lipoprotein decrease cholesterol efflux from human cultured fibroblasts. Biochem Biophys Acta 1992;1125:230-235.

44. Guertin F, Brunet S, Gavino V, Tuchweber B, Levy E. Malondialdehyde-Modified HDL Leads to Accumulation of Cholesterol in Rat Liver Endothelial Cells. Biochem Biophys Res Commun 1995;212:1-8.

45. Shao B, Pennathur S, Pagani I, Oda MN, Witztum JL, Oram JF, Heinecke JW. Modifying apolipoprotein A-I by malondialdehyde, but not by an array of other reactive carbonyls, blocks cholesterol efflux by the ABCA1 pathway. J Biol Chem 2010;285:18473-18484.

46. Ferretti G, Bacchetti T, Moroni C, Savino S, Liuzzi A, Balzola F, Bicchiega V. Paraoxonase activity in high-density lipoproteins: A comparison between healthy and obese females. J Clin Endocrinol Metab 2005;90:1728-1733.

47. Bhattacharyya T, Nicholls SJ, Topol EJ, Zhang R, Yang X, Schmitt D, Fu X, Shao M, Brennan DM, Ellis SG, Brennan ML, Allayee H, Lusis AJ, Hazen SL. Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. JAMA 2008;299:1265-1276.

48. Regieli JJ, Jukema JW, Doevendans PA, Zwinderman AH, Kastelein JJ, Grobbee DE, van der Graaf Y. Paraoxonase variants relate to 10-year risk in coronary artery disease: Impact of a high-density lipoprotein–bound antioxidant in secondary prevention. J Am Coll Cardiol 2009;54:1238-1245.

49. Sankaranarayanan S, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Asztalos BF, Bittman R, Rothblat GH. A sensitive assay for ABCA1-mediated cholesterol efflux using BODIPY-cholesterol. J Lipid Res 2011;52:2332-2340.

Figure Legends

Figure 1: HAEC were incubated with HDL (50 µg/ml) isolated from healthy controls (healthy) at begin (Beg) and after performing an exercise training program (end), CHF patients in NYHA-II (NYHA II) and CHF patients in NYHA III at begin (Beg) and after finishing of an exercise training program (End) and the phosphorylation of eNOS at position Ser^{1177} (A) and Thr^{495} (B) was evaluated by western-blot analysis. To compensate for protein loading differences phosphorylation at the specific site was normalized to the non-phosphorylated form. For quantitative analysis the x-fold increase in eNOS phosphorylation of HDL incubated cells vs. untreated cells was determined. Representative examples of the western blots are depicted on top of the figure. Values are expressed as mean±SEM.

Figure 2: Effect of HDL (50 µg/ml, 60 minutes, 37°C) from healthy controls (healthy) before (Beg) and after an exercise programme (end), CHF patients in NYHA-II (NYHA II) and CHF patients in NYHA III at begin (Beg) and after finishing of ET (End) on endothelial NO production as determined by ESR spectroscopy. Data are expressed as percent change versus buffer-treated cells. Values are expressed as mean±SEM.

Figure 3: HAEC were incubated with HDL (50 µg/ml) isolated from healthy controls (healthy) at begin (beg) and after finishing an ET program (end), CHF patients in NYHA-II (NYHA II) and CHF patients in NYHA III at begin (Beg) and after finishing an ET program (end) and the phosphorylation of PKC-ßII at position Ser^{660} was evaluated by western-blot analysis (A). HAEC were incubated with HDL (50 µg/ml) isolated from CHF-NYHA-IIIb patient in the presence or absence of a PKC-ßII inhibitor (+/- CG53353) and the phosphorylation of eNOS-Ser^{1177} (B) and eNOS-Thr^{495} (C) was evaluated. To compensate for protein loading differences phosphorylation at the specific site was normalized to the

non-phosphorylated form. For quantitative analysis the x-fold increase in PKC-ßII phosphorylation of HDL incubated cells vs. untreated cells was determined. Representative examples of the western blots are depicted on top of the figure. Values are expressed as mean±SEM.

Figure 4: HAEC were incubated with HDL (50 µg/ml) isolated from healthy controls (healthy) at begin (beg) and after finishing an ET program (end), CHF patients in NYHA-II (NYHA II) and CHF patients in NYHA III at begin (Beg) and after finishing an ET program (end) and the phosphorylation of p70S6K at position Ser^{411} was evaluated by western-blot analysis (A). HAEC were incubated with HDL (50 µg/ml) isolated from CHF-NYHA-IIIb patient in the presence or absence of a PKC-ßII inhibitor (+/- CG53353) (B) or the p70S6K inhibitor rapamycin (+/- rapamycin, C,D) and the phosphorylation of p70S6K-Ser^{411} (B) or the the phosphorylation of eNOS-Ser^{1177} (C) and eNOS-Thr^{495} (D) was evaluated respecively. To compensate for protein loading differences phosphorylation at the specific site was normalized to the non-phosphorylated form. For quantitative analysis the x-fold increase in PKC-ßII phosphorylation of HDL incubated cells vs. untreated cells was determined. Representative examples of the western blots are depicted on top of the figure. Values are expressed as mean±SEM.

Figure 5: Quantification of protein-bound MDA in HDL isolated from healthy controls (healthy), CHF patients in NYHA-II (NYHA II) and CHF patients in NYHA III (A) as well as the change after performing an exercise training programme (B). Pon 1 paraoxonase activity was measured in HDL isolated from healthy controls (healthy) before (Beg) and after an ET program (end), CHF patients in NYHA-II (NYHA II) and CHF patients in NYHA III at begin (Beg) and after finishing an ET program (end) (C). In addition, the total amount lipid peroxides as measure for the oxidative load was quantified in the plasma of all study

participants (D). Values are expressed as mean±SEM.

Figure 6: A schematic drawing depicting common and unique proteins between the analyzed groups (A). A principal component analysis plot to separate HDL from healthy and CHF-NYHA-IIIb as well as before (Beg) and after performing an exercise training program (end) (B). A Volcano plot shows the significance versus effect size-change to quickly visualize the most meaningful changes comparing $HDL_{healthy}$ and $HDL_{NYHA\text{-}IIIb}$ at begin (C) and after the exercise training program (C).

Figure 7: Hypothetical working model, how disease progression and ET influences HDL-induced NO production. Disease progression leads to a higher amount of MDA bound to HDL, whereby PKC-ßII gets more and more activated. This activation leads to an inhibition of HDL-mediated NO production due to a reduced eNOS phosphorylation at eNOS-Ser^{1177} and a higher phosphorylation at eNOS-Thr^{495}. This process seems to be partial reversible by ET.

Table 1: Clinical characteristics and cardiovascular medication

	Healthy Begin (n=8)	Healthy End (n=8)	CHF NYHA-II (n=8)	CHF NYHA-IIIb Begin (n=8)	CHF NYHA-IIIb End (n=8)
Age [years]	67±4		54±4	63±3	
Body mass index [kg/cm^2]	26.9±0.9		30.6±1.5	26.9±0.9	
Arterial hypertension [n]	x (xx%)		3 (37.5%)	7 (87.5%)	
Diabetes mellitus [n]	x (xx%)		4 (50.0%)	3 (37.5%)	
Active smoking [n]	x (x%)		0 (0%)	0 (0%)	
LV ejection fraction [%]	60±2		26±2 **	24±2 **	34±2 †
Peak VO_2 [ml/kg*min]	xx±xx		17.6±1.1 *	14.3±1.2 **	17.4±1.6 †
Etiology of heart failure					
Ischemic heart disease [n]	- -		3 (37.5%)	4 (50.0%)	
Dilative cardiomyopathy [n]	- -		5 (62.5%)	4 (50.0%)	
Cardiovascular medication					
Beta blocker [n]	1 (12.5%)		5 (62.5%) *	7 (87.5%) *	
ACE inhibitor or ATII blocker	3 (37.5%)		8 (100%) *	7 (87.5%) *	

Formatiert: Englisch (USA)

[n]					
Aldosterone antagonist [n]	0 (0%)		3 (37.5%) *	8 (100%) *	
Lipid profile					
Total cholesterol [mmol/L]	6.4±0.4		6.3±0.3	5.3±0.6	4.7±0.3
LDL cholesterol [mmol/l]	3.5±0.3		4.3±0.4	3.4±0.5	2.7±0.3
HDL cholesterol [mmol/L]	1.7±0.3		1.4±0.1	1.1±0.1 *,§	1.2±0.1

Data are mean±SEM. Abbreviations: LV: left ventricular; peak VO_2: peak oxygen consumption; CHF: chronic heart failure; ACE inhibitor: angiotensin converting enzyme inhibitor; ATII blocker: angiotensin II subtype I receptor blocker; LDL: low density lipoprotein; HDL: high density lipoprotein. * $p<0.05$ vs. healthy; ** $p<0.001$ vs. healthy; § $p<0.05$ vs. CHF-NYHA-II; † $p<0.05$ vs. CHF-NYHA-IIIb Beg.

Material and Methods

NanoLC-MS/MS analysis

The proteome of isolated HDL particles from 7 NYHA-IIIb patients and 8 healthy controls before and after an ET program were investigated by shotgun LC-MS/MS. In total, 4 µg of protein lysates were reduced (2.5 mM DTT for 1 h at 60°C) and alkylated (10 mM iodacetamide for 30 min at 37°C). Proteolysis was performed overnight using trypsin (Promega, Madison, WI) with a ratio of 1:25 at 37°C. The tryptic digestion was stopped by adding acetic acid at the final concentration of 1%, followed by desalting and purification using ZipTip-µC18 tips (Millipore, Billerica, MA).

Proteolytically cleaved peptides (500 ng) were, prior to mass spectrometric analyses, enriched on a 2 cm Acclaim PepMap100-precolumn (C18 2 µm, 100 Å) and then separated by reverse phase nano HPLC on a 15 cm Acclaim PepMap RSLC-column (C18 2 µm, 100 Å) using an Dionex UltiMate 3000 RSLCnano system (Thermo Scientific, Waltham, MA) at a constant flow rate of 300 nL/min. Separation was achieved using a linear gradient of 60 min (2%-25%) with 0.1% acetic acid, 2% acetonitrile in water (solvent A) and 0.1% acetic acid in 100% acetonitrile (solvent B). Separated peptides were monitored using a QExactive mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source (Advion BioSystems, Ithaca, NY). The MS-instrument was operated in data-dependent acquisition (DDA) mode. MS settings were as follow: survey full-scan spectra were acquired with a resolution R = 70,000, automated gain control (AGC) target was set to 3e6 ions, the maximum injection time was set to 250 ms. MS/MS scan events were repeated for top 10 peaks

using the higher energy dissociation mode (HCD) at normalized collision induced energy of 27.5%, underfill ratio (5%) with an intensity threshold of 8.3e4 ions was selected. Already targeted ions for MS/MS were dynamically excluded for 30 s with monoisotopic precursor selection enabled. Raw data from the QExactive instrument was processed using the Refiner MS 7.5 and Analyst 7.5 module (Genedata, Basel, Switzerland). Refiner MS performed peak detection, noise analysis, peak integration, isotope grouping and retention time alignment of the LC-MS/MS data. Generated peak lists were searched against a human FASTA-formatted database containing 20,268 unique entries (human_uniprot_swissprot_2011_10.fasta) using an in-house Mascot server v2.3.2 (Matrix Science, London, GB). Database searches were performed with carbamidomethyl on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion tolerance and 20 mmu MS/MS tolerance. Only ranked one peptide hits and a Mascot ion score >23 were considered as identified. After peak annotation, the data were further processed in Analyst 7.5, where statistical data evaluation was performed using univariate and multivariate methods. Paired sample T-Test and parametric T-test were used for group comparison. A value of $p<0.05$ was considered as statistically significant. A Principal Components analysis (PCA) was applied as classical means of dimensionality reduction and visualization of multivariate data. Proteins were functionally assigned to canonical pathways using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA).

Figure Legends.

Supplementary figure 1: Correlation analysis between the HDL-induced eNOS phosphorylation at Ser^{1177} (A) or change in absolute NO production (B) and the change in endothelial function due to the 3 month of exercise intervention.

Table 1: Proteins identified by label free shotgun LC-MS/MS analysis

Protein name	UNI Prot Accesion Numbers	Description	number of identified peptides	control vs. NYHA baseline	control vs. NYHA post	control pre vs. post	NYHA pre vs. post
A1AT	P01009	Alpha-1-antitrypsin	22	0.019 (1.77)	0.003 (2.09)	0.087	0.792
CO3	P01024	Complement C3	17	0.394	0.018 (-0.72)	0.843	0.083
APOM	O95445	Apolipoprotein M	9	0.01 (0.84)	0.006 (0.76)	0.435	0.846
APOC3	P02656	Apolipoprotein C-III	7	0.001 (-1.45)	0.001 (-1.1)	0.137	0.432
APOL1	O14791	Apolipoprotein L1	7	0.038 (-0.91)	0.111	0.245	0.87
APOC4	P55056	Apolipoprotein C-IV	5	0.001 (-1.62)	0.001 (-1.7)	0.644	0.588
SAA	P02735	Serum amyloid A protein	5	0.759	0.007 (-2.3)	0.094	0.287
CNDP1	Q96KN2*	Beta-Ala-His dipeptidase	4	0.606	0.028 (0.75)	0.085	0.844
CO5	P01031	Complement C5	4	0.033 (-0.85)	0.109	0.841	0.538
APOC2	P02655	Apolipoprotein C-II	3	0.003 (-0.89)	0.005 (-0.84)	0.004 (0.19)	0.496
HPTR	P00739	Haptoglobin-related protein	3	0.032 (1.67)	0.496	0.382	0.66
A2MG	P01023	Alpha-2-macroglobulin	2	0.365	0.003 (-2.46)	0.088	0.823
HPT	P00738	Haptoglobin	2	0.017 (-1.87)	0.747	0.135	0.304
PCSK9	Q8NBP7	Proprotein convertase subtilisin/kexin type 9	2	0.072	0.002 (-1.12)	0.63	0.397
THRB	P00734	Prothrombin	2	0.003 (4.97)	0.001 (4.81)	0.228	0.344
ABCC8	Q09428*	ATP-binding cassette sub-family C member 8	1	0.08	0.014 (2.3)	0.532	0.683
BPIB1	Q8TDL5	BPI fold-containing family B member 1	1	0.089	0.023 (-0.34)	0.296	0.239
DHX8	Q14562*	ATP-dependent RNA helicase DHX8	1	0.017 (2.4)	0.003 (2.89)	0.323	0.549
HRG	P04196	Histidine-rich glycoprotein	1	0.038 (2.11)	0.002 (2.37)	0.202	0.81
LBP	P18428	Lipopolysaccharide-binding protein	1	0.037 (-1.01)	0.002 (-1.52)	0.697	0.207
SLIT1	O75093*	Slit homolog 1 protein	1	0.256	0.042 (-0.83)	0.280	0.325

* novel identified proteins; group-wise comparison based on independent or dependent (paired) student T-Test. Data were represented by p-Value (effect size)